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Running head: diatom in elevated CO_2 over 1800 generations

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Primary Research Article

Abstract

Studies on the long-term responses of marine phytoplankton to ongoing ocean acidification (OA) are appearing rapidly in the literature. However, only a few of these have investigated diatoms, which is disproportionate to their contribution to global primary production. Here we show that a population of the model diatom *Phaeodactylum tricornutum*, after growing under elevated CO_2 (1000 μatm, HCL, pH_T: 7.70) for 1860 generations, showed significant differences in photosynthesis and growth from a population maintained in ambient CO_2 and then transferred to elevated CO_2 for 20 generations (HC). The HCL population had lower mitochondrial respiration, than did the control population maintained in ambient CO_2 (400 μatm, LCL, pH_T: 8.02) for 1860 generations. Although the cells had higher respiratory carbon loss within 20 generations under the elevated CO_2, being consistent to previous findings, they down-regulated their respiration to sustain their growth in longer duration under the OA condition. Responses of phytoplankton to OA may depend on the timescale for which they are exposed due to fluctuations in physiological traits over time. This study provides the first evidence that populations of the model species, *P. tricornutum*, differ phenotypically from each other after having been grown for differing spans of time under OA conditions, suggesting that long-term changes should be measured to understand responses of primary producers to OA, especially in waters with diatom-dominated phytoplankton assemblages.

Introduction

Diatoms play a critical role in the carbon cycle as they are responsible for about 20% of global primary production (Field *et al.*, 1998). They contribute about 35% to the primary productivity of the oligotrophic oceans and nearly 75% to that of the coastal zone and other nutrient-rich systems (Nelson *et al.*, 1995). In addition, due to the presence of a silicon frustule, diatoms are key species influencing the biogeochemical cycle of silicon (Si); indeed, every atom of silicon delivered to the ocean is incorporated into a diatom cell wall on average 39 times before being removed to the seabed (Treguer *et al.*, 1995). Ongoing global climate changes, such as ocean acidification (OA) as a consequence of increasing anthropogenic CO_2 release (Sabine *et al.*, 2004; Orr *et al.*, 2005), are suggested to have consequences for marine plankton (Doney *et al.*, 2009; Riebesell & Tortell, 2011) and carbon and elemental cycles, which will feed back to global climate.

Positive, negative, and even neutral effects of OA on diatoms have been documented (Gao & Campbell, 2014). On one hand, OA may enhance growth and photosynthesis, and down-regulate the CO_2 concentrating mechanisms (CCMs); on the other hand, it can increase carbon loss mechanisms, including respiration and photorespiration (Gao *et al.*, 2012b), though decreased mitochondrial respiration has also been reported (Hennon *et al.*, 2014). Moreover, the decreased seawater pH induced by increasing dissolved CO_2 from the atmosphere may impose a higher energy cost for phytoplankton cells to maintain intracellular acid-base homeostasis (Raven, 2011) and may also influence nutrient availabilities and toxicities (Millero,

2009). OA can also influence the effects of other environmental factors on phytoplankton cells. For example, photoinhibition under high light or in the presence of UV radiation is greater under OA conditions (Sobrino *et al.*, 2008; Wu *et al.*, 2010; Chen & Gao, 2011; Sobrino *et al.*, 2014). Nevertheless, OA may mitigate the deleterious effects of UVB, as shown by Li *et al.* (2012b) for a diatom. Species-specific (Langer *et al.*, 2006; Kroeker *et al.*, 2010), and even strain-specific (Langer *et al.*, 2009), responses and the impact of other factors, such as light intensity (Gao *et al.*, 2012b), nutrient concentration (Verspagen *et al.*, 2014), and temperature (Fu *et al.*, 2007; Feng *et al.*, 2009), may partially explain the lack of conformity in the reported ecological and physiological effects of OA. Whether phytoplankton cells will benefit from increased availability of CO_2 with progressive OA thus depends on the balance of effects under multiple stressors in different regions (Riebesell & Gattuso, 2015).

Most prior studies have been based on short-term experiments. Limited knowledge has thus been documented on long-term responses of marine phytoplankton to OA. Several recent reviews emphasize the importance of long-term exposure research as one of the major directions for future OA studies (Gao *et al.*, 2012a; Reusch & Boyd, 2013; Gao & Campbell, 2014; Sunday *et al.*, 2014; Riebesell & Gattuso, 2015). Since the earliest publication of evolutionary responses to elevated CO_2, based on a green alga (Collins & Bell, 2004), the literature in this field has increased rapidly, mainly focusing on coccolithophore species (Müller *et al.*, 2010; Lohbeck *et al.*, 2012; Benner *et al.*, 2013; Jin *et al.*, 2013; Lohbeck *et al.*,

2013; Lohbeck *et al.*, 2014). At this time, only a few studies have investigated responses of diatoms to OA over long periods (Crawfurd *et al.*, 2011; Low-Décarie *et al.*, 2013; Tatters *et al.*, 2013). In the present study, we grew a model species of diatom, *Phaeodactylum tricornutum*, for 1860 generations under ambient and elevated CO_2 conditions along with controlled carbonate chemistry, and investigated growth and physiological differences between short- and long-term CO_2 exposure populations.

Materials and methods

Culture conditions

Phaeodactylum tricornutum (CCMA 106) was originally isolated from the South China Sea (SCS) in 2004 and obtained from the Center for Collections of Marine Algae (CCMA) of the State Key Laboratory of Marine Environmental Science, Xiamen University. Cells were incubated in autoclaved natural seawater collected from the coastal region of Quanzhou, China (118.60°E, 24.52°N). Nutrients were added according to the recipe for Aquil medium (Morel *et al.*, 1979). The incubation light intensity was 150 μmol photons m^{-2} s^{-1}, provided by cool white fluorescent tubes. Cultures were maintained at 20 ^{o}C with a 12h : 12h light dark cycle. Polycarbonate bottles used for culturing cells were placed in one incubator. They were manually shaken once a day and their positions relative to the light source were randomly changed, so that cells were not systematically exposed to different levels of illumination. The pH was measured before and after dilution by a pH meter

(Orion 2 STAR, Thermo Scientific) calibrated with standard National Bureau of Standards (NBS) buffers. CO_2 partial pressure was measured by a CO_2 detector (M170, Vaisala Oyj). Carbonate chemistry parameters were calculated by CO2SYS software using pH and pCO_2. Values of pH and pCO_2 were measured periodically (but not at every dilution due to logistical difficulties) during the long-term exposure. Cell density was maintained below 1.2×10^5 per ml (exponential growth phase) and the carbonate chemistry remained stable over the culture period, with pH variations < 0.08 units. Carbonate chemistry of media at the end of experiment are shown in Table S1.

Experimental set-up

For the long-term experiment, Aquil medium was pre-aerated either with ambient outdoor air (400 μatm pCO_2) for the long-term ambient air treatment (hereafter termed LCL) or with CO_2 enriched air (1000 μatm pCO_2) for long-term CO_2 enriched air treatment (hereafter termed HCL) before dilution. The latter condition was achieved within a CO_2 plant growth chamber (HP1000G-D, Ruihua), in which CO_2 partial pressure was continuously monitored and maintained at 1000 ± 50 μatm. The cultures were diluted every 5 to 7 days to maintain a stable carbonate system within the LCL and HCL treatments. Three separate bottles were maintained during each growth cycle (5 ~ 7 days), and then the triplicate cultures of LCL or HCL treatment were pooled by treatment at the end of the growth cycle, and then diluted to separate bottles with fresh medium equilibrated with the CO_2 levels. This means

that there is a single population for each treatment, and triplicate bottles were used to measure growth rate and cell size of each population in the growth cycle. Cells were grown under LCL and HCL conditions for 1035 days (about 1860 generations) before having their traits measured.

To investigate short-term OA effects, LCL cells were inoculated into the medium pre-aerated with 1000 μatm pCO_2 air at the end of the long-term experiment above and grown for another 20 generations (HC treatment) before sampling. LCL cells rather than ancestral cells were used so that the entire experiment could be measured at once to avoid time-block effects, so the LCL population growing in air is the "control" phenotype to which the short- and long-term phenotypes (HC and HCL) are compared. Subsamples for measurement of physiological parameters were always taken in the middle of the light period to avoid diurnal variations (Ragni & Ribera d'Alcalà, 2007).

Specific growth rate and mean cell size determination

Cell concentration and mean cell size were measured by a Coulter Particle Count and Size Analyzer (Z2, Beckman Coulter). Specific growth rate was calculated according the equation: $\mu = (\ln N_1 - \ln N_0)/(t_1 - t_0)$, in which N_1 and N_0 represent cell concentrations at t_1 and t_0.

Chlorophyll and carotenoid contents

Cells for determination of pigment contents were filtered onto GF/F filters (25 mm,

Whatman), and then extracted overnight in absolute methanol at 4 °C in darkness. After centrifugation (5000 g for 10 min), the absorption values of the sample supernatant were analyzed by a UV-VIS Spectrophotometer (DU800, Beckman Coulter). The concentrations of chlorophylls *a* and *c* were calculated according to Ritchie (2006):

Chl *a* ($\mu g\ ml^{-1}$) = $13.2654 \times (A_{665} - A_{750}) - 2.6839 \times (A_{632} - A_{750})$;

Chl *c* ($\mu g\ ml^{-1}$) = $-6.0138 \times (A_{665} - A_{750}) + 28.8191 \times (A_{632} - A_{750})$.

Carotenoid concentration was determined by the equation given by Strickland & Parsons (1972):

Carotenoid ($\mu g\ ml^{-1}$) = $7.6 \times ((A_{480} - A_{750}) - 1.49 \times (A_{510} - A_{750}))$, where Ax indicates the absorbance at a wavelength x. The pigment content per cell was calculated by taking the dilution factor and cell concentration into account.

Chlorophyll *a* fluorescence parameters

In vivo chlorophyll *a* fluorescence parameters of *P. tricornutum* cells were determined using a Xenon-Pulse Amplitude Modulated fluorometer (Xe-PAM, Walz). Maximum and effective photochemical quantum yields were determined according to the equations of Genty *et al.* (1989): maximum photochemical quantum yield, $\Phi_{PSII\ max} = (F_m - F_0) / F_m$ for dark-adapted (10 min) samples; effective photochemical quantum yield, $\Phi_{PSII\ eff} = (F'_m - F_t) / F'_m = \Delta F / F'_m$ for light-adapted samples, where F_m and F_m' indicate maximum chlorophyll fluorescence of dark and growth-light-adapted samples, respectively; F_0 is the minimum chlorophyll

fluorescence of dark-treated cells; and F_t is the steady state chlorophyll fluorescence of light-exposed samples. Non-photochemical quenching (NPQ) was calculated as: $NPQ = (F_m - F'_m) / F'_m$. The saturation pulse was set at 5000 μmol photons m^{-2} s^{-1}, and lasted for 0.8 s.

Rapid light curves (RLCs) were measured to evaluate relative maximum electron transport rate ($rETR_{max}$), apparent photon transfer efficiency (α), and light saturation point (I_k).Values of rETR under eight actinic, progressively increasing, light intensities were assessed as previously reported (Wu *et al.*, 2010): $rETR = PAR \times \Phi_{PSII\ eff} \times 0.5$, where PAR represents the photon flux density of actinic light (μmol photons m^{-2} s^{-1}), Φ_{PSII} is the effective photochemical quantum yield, and the factor 0.5 is based on the assumption that PSII receives half of all absorbed quanta. Before the RLC measurements, samples were incubated at 150 μmol photons m^{-2} s^{-1} under 20 °C (i.e. growth conditions) for 10 min to avoid the influence of quasi-dark light conditions on rETR during manipulation. RLCs were fitted to the following model: $P = PAR / (a \times PAR^2 + b \times PAR + c)$, where P is rETR, PAR is photon flux density of actinic light (μmol photons m^{-2} s^{-1}), and a, b, c are model parameters. I_k, $rETR_{max}$, and α were calculated from a, b, and c according to Eilers & Peeters (1988). The relative photoinhibition ratio for rETR was evaluated by the following equation: $Inh\ (\%) = (rETR_{max} - rETR_x) / rETR_{max} \times 100\%$, where $rETR_x$ represents rETR at 1590 μmol photons m^{-2} s^{-1} actinic light intensity.

Photosynthesis and respiration measurements

Net photosynthetic oxygen evolution was determined by a Clark-type oxygen electrode (Hansatech) at 20 °C and 150 μmol photons m^{-2} s^{-1}. The temperature was controlled by a refrigerated circulating bath (GDH-0506, Shunma). In the middle of the light period, cells were concentrated by gently filtering (< 0.02 MPa) them onto a 47 mm cellulose acetate membrane (1 μm pore-size, Xinya), and they were then re-suspended into 20 mmol L^{-1} Tris buffered medium whose pH was pre-adjusted by freshly prepared hydrochloric acid and sodium hydroxide to their corresponding culture medium values. Use of Tris-buffer was necessary to maintain the appropriate pH at the higher cell densities used for oxygen evolution experiments. Samples with a known concentration around 2×10^6 cells per milliliter were injected into the oxygen electrode chamber, and were magnetically stirred. Samples for dark respiration determination were filtered and re-suspended in the same way as mentioned above, and oxygen consumption rates were monitored in the dark. Rates of oxygen evolution and consumption were recorded when they became constant. Additionally, photosynthetic rates of LCL and HCL populations were determined by the ^{14}C method (see supporting information for detail) at day 558 and 588.

The population responses to elevated CO_2

Responses to elevated CO_2 of individual parameters for the HC or HCL population were calculated as the fold difference compared to the LCL control. For both population responses, a value of 1 indicates no response, a value < 1 indicates a negative response and a value > 1 indicates a positive response to elevated CO_2. The

value was set to 1 when no significant difference was detected between HC or HCL and LCL populations.

Statistical analyses

Triplicate cultures were used for each population (LCL, HC, and HCL), and the data are expressed as means and standard deviations for each population. One-way ANOVA and Tukey tests were used for analyzing significant differences in parameters among populations (LCL, HC, and HCL) at the end of the experiment with 95% confidence intervals. Significant differences in growth rates and cell sizes between LCL and HCL populations for every measured growth cycle from days 503 to 1035 were analyzed using independent samples t tests with 95% confidence intervals. Specific growth rates and cell sizes of both populations for every measured growth cycle and p values of independent t tests are shown in Tables S2 and S3.

Results

Growth rate and mean cell size

The specific growth rates ranged between $0.96 \pm 0.11 \sim 1.50 \pm 0.01$ d^{-1} in both LCL and HCL populations over the entire experiment. From days 503 to 960, 33 of the 54 growth cycles showed no significant differences in growth rate between LCL and HCL populations (Fig.1a & Table S2, independent t test, $p > 0.05$). No consistent trend was found in the other 21 growth cycles which showed significant differences between populations, with the exceptions of growth rates from days 568 to 588

where cells from the HCL population showed a slower growth rate than cells from LCL population over the 20 days (independent t test, $p < 0.05$). However, this trend disappeared from day 589 to day 960. From days 960 to 1035, there was a 10% decrease of growth rate of HCL relative to the LCL population (independent t test, $p < 0.05$). After having been grown for 1035 days (about 1860 generations), the specific growth rate of cells from the HCL population was 13% lower than cells from the LCL population (Fig. 2a, one-way ANOVA, $p < 0.001$). In contrast, no difference was detected in growth rates between LCL and HC populations (one-way ANOVA, $p = 0.133$).

Mean cell sizes (width) ranged from $4.6 \pm 0.02 \sim 5.6 \pm 0.02$ μm during the entire experiment, and the differences between the two populations varied over time. Between days 503 and 649, cells in 12 of the 20 growth cycles showed statistically similar cell sizes between populations (Fig. 1b & Table S3, independent t test, $p > 0.05$), and no consistent trend was found in the other 8 growth cycles. From days 649 to 1035, 25 of the 31 growth cycles showed that HCL cells were 3.4% smaller than LCL cells (independent t test, $p < 0.05$). At the end of the entire experiment, HCL cells were 3% smaller than LCL cells (Fig. 2b, one-way ANOVA, $p < 0.001$), and no differences were observed between LCL and HC populations (one-way ANOVA, $p = 0.863$).

Chlorophyll and carotenoid contents

After 1860 generations (1035 days) of growth, the chlorophyll *a* content of LCL

cells was 0.27 ± 0.01 pg cell^{-1}, and HCL cells showed a 35% increase, reaching 0.36 ± 0.05 pg cell^{-1} (Fig. 2c, one-way ANOVA, p = 0.021). Cells from the HC population had a chlorophyll *a* content of 0.22 ± 0.01 pg cell^{-1}, and no significant difference was found between LCL and HC populations (one-way ANOVA, p = 0.206). Similarly, the carotenoid content of HCL cells was 40% higher than that in the LCL population (Fig. 2d, one-way ANOVA, p = 0.021). Carotenoid contents of LCL and HC populations were not statistically different. There was no significant difference in chlorophyll *c* content among populations, with a value about 0.04 ± 0.01 ~ 0.06 ± 0.02 pg cell^{-1}.

In vivo chlorophyll *a* fluorescence parameters

$\Phi_{PSII\ max}$ values of *P. tricornutum* cells were similar in all populations, with a value of 0.672 ± 0.007 (Table 1). There was no difference in effective photochemical quantum yield ($\Phi_{PSII\ eff}$) between LCL and HCL cells, while HC cells showed a 3% enhancement of $\Phi_{PSII\ eff}$ compared to LCL cells (one-way ANOVA, p = 0.009), $\Phi_{PSII\ eff}$ of HC cells reached 0.618 ± 0.003 after illumination with actinic light close to the culture light intensity. As a result, rETR under growth light was stimulated by 3% in the HC population compared with the LCL cells. For NPQ, no significant difference was observed among populations.

I_k obtained from RLCs showed no differences among LCL, HCL, and HC populations. Values for $rETR_{max}$ of the HCL cells were 4% lower than those of cells in the LCL and HC populations (one-way ANOVA, p = 0.025, p = 0.036,

respectively). Photoinhibition of *P. tricornutum* cells observed in RLCs was obvious in all three populations. HC and HCL cells showed higher photoinhibition ratios than LCL ones (one-way ANOVA, $p = 0.004$, $p = 0.020$, respectively), though no statistical difference was found between HC and HCL cells. Values for inhibition ratios of LCL, HCL, and HC were 11.4 ± 0.02%, 16.9 ± 0.01%, and 19.2 ± 0.02%, respectively. There were no significant differences in α found among populations.

Photosynthesis and dark respiration

Chlorophyll-normalized dark respiration under the corresponding growth conditions showed differing patterns: the HC population had a 179% enhancement of dark respiration compared to the LCL population (Fig. 3a, one-way ANOVA, $p < 0.001$). However, dark respiration in the HCL population was statistically the same as that of cells from the LCL population (one-way ANOVA, $p = 0.069$). Net photosynthetic oxygen evolution rates per chl *a* were 0.56 ± 0.10, 0.36 ± 0.06, and 0.73 ± 0.07 μmol (μg chl *a*)$^{-1}$ h^{-1} for LCL, HCL, and HC populations (Fig. 3c), respectively. HCL cells showed 35.7% and 50.7% lower net photosynthetic rate than LCL and HC ones, respectively (one-way ANOVA, $p = 0.049$, $p = 0.003$).

When normalized to per cell, the HC population had a 129% increase of dark respiration per cell compared to cells of LCL population (Fig. 3b, one-way ANOVA, $p < 0.001$), and the rate was reduced by 41.9% in the HCL population relative to the LCL population (one-way ANOVA, $p = 0.048$). There were no statistically significant differences for net oxygen evolution per cell among populations (Fig. 3d).

Photosynthetic carbon fixation rates were 1.32 ± 0.09 and 1.46 ± 0.10 pg cell^{-1} h^{-1} at day 558 and 588, respectively (Fig. S1). No significant differences in photosynthetic rate were found between LCL and HCL populations (one-way ANOVA, $p > 0.05$).

The population responses to elevated CO_2

Differences between the HC and HCL populations are summarized in Table 2. The HC population showed no response of growth rate, cell size, chlorophyll *a*, and photosynthetic rate to elevated CO_2. The dark respiration of the HC population showed a positive response to elevated CO_2. In contrast, the HCL population showed a differing pattern: values for growth rate, cell size, dark respiration, and chlorophyll-normalized photosynthesis were all below 1, and the value for chlorophyll content was greater than 1.

Discussion

Previous studies have shown that elevated CO_2-induced OA either stimulates (Wu *et al.*, 2010; Gao *et al.*, 2012b; Li *et al.*, 2014), or shows no measurable effect on (Li *et al.*, 2012a; Wu *et al.*, 2014), growth in *P. tricornutum* under low light (the intensity ranged from 60 ~ 460 μmol photons m^{-2} s^{-1} for constant indoor light; the mean intensity value ranged from 50 ~180 μmol photons m^{-2} s^{-1} for fluctuating outdoor solar radiation) on time scales of under tens of generations. Here, we found that a population of the same strain grown for over a thousand generations in OA

conditions had decreased growth, along with decreased cell size and mitochondrial respiration. This demonstrates the potential for a negative long-term response to OA in this species, despite multiple studies showing a positive short-term response. The population grown for > 1800 generations in high CO_2 shows a syndrome outside the range of those reported for this species after short-term exposure to elevated CO_2. For example, in terms of carbon balance, cells under short-term OA tended to increase photosynthetic rate per chl *a* (i.e. a lower input of chlorophyll build-up, but a higher output of photosynthesis per chl *a* than HCL cells); while the population grown under long-term OA reduced carbon expenditure (i.e. decreasing cell size and dark respiration) to remedy the lower photosynthetic rate. While the observation of a single outlier population does not conclusively show that the long-term response differs from the short-term one in this species, it does show that the possible long-term (probably evolutionary) responses fall outside the range of reported short-term responses for this species, confirming both the value of, and need for, further experiments on the evolutionary potential of marine diatoms.

Photosynthesis and dark respiration were both enhanced in *Thalassiosira pseudonana* (Yang & Gao, 2012) and *P. tricornutum* (Wu *et al.*, 2010) when grown under OA for a short period. However, *T. pseudonana* cultured for tens of generations decreased both photosynthesis and respiration under elevated CO_2 and nitrate limitation (Hennon *et al.*, 2014), while dark respiration of Southern Ocean diatoms remained unaltered or decreased (depending on species) under the habitat-relevant low temperature after two-weeks OA acclimation (Trimborn *et al.*,

2014). OA exposure for less than 30 days usually enhances primary production or dissolved inorganic carbon drawdown by phytoplankton assemblages (Hein & Sand-Jensen, 1997; Riebesell *et al.*, 2007; Egge *et al.*, 2009; Engel *et al.*, 2013), though it decreased primary productivity in the South China Sea (Gao *et al.*, 2012b). Such an enhancement or inhibition caused by OA has been attributed to the saved energy due to down-regulation of CCMs under the elevated pCO_2 (Raven, 1991; Wu *et al.*, 2010; Hopkinson *et al.*, 2011). Nevertheless, no detectable effects of OA on primary production were found in Antarctic phytoplankton assemblages grown under 50% incident solar radiation, with the CCMs being down-regulated (Young *et al.*, 2015). By now, it is clear that species-specificity, alongside interactions between environmental drivers, contributes to variations in the responses of species or communities to OA.

The decreased chlorophyll-normalized photosynthesis of cells in the long-term high CO_2 population was due to their higher chlorophyll *a* content, since their photosynthesis per cell was similar to LCL cells. The higher pigment content may in turn lead to an increased package effect, i.e., self-shading. Moreover, the down-regulation of carbonic anhydrase (CA) and Rubisco expression and activities under high CO_2 conditions (Aizawa & Miyachi, 1986; Tortell *et al.*, 2000; Hopkinson *et al.*, 2013; Losh *et al.*, 2013) may also be involved in depressing photosynthesis in the HCL population. As a result, cells from the HCL population had a lower photosynthetic rate per chl *a* than cells from LCL and HC populations.

Fluctuations of cell size in both populations were observed during the long-term

exposure, which might be related to periods of auxospore production by the diatom. While HCL and LCL populations showed similar variation patterns, HCL cells became smaller over 700 days compared to LCL ones, which may be a general trend in phytoplankton exposed to elevated CO_2 over long periods of time (Collins *et al.*, 2014). The smaller cell size of the HCL population, which could be attributed to down-regulations of physiological performance (growth, photosynthesis, and respiration), could be a strategy to reduce carbon expenditure. The carbon saved by decreased respiration or decreases in some cellular components may be allocated to other components such as light harvesting systems (indicated by higher pigment contents in the HCL population). Both the generality and underlying mechanisms of these trends require further investigation.

In long-term OA incubations of other species, OA has been found to stimulate (Low-Décarie *et al.*, 2013; Tatters *et al.*, 2013), depress (Tatters *et al.*, 2013) or have no detectable effect (Crawfurd *et al.*, 2011) on the growth rates of diatoms. In addition, other studies show that selection on growth rate in OA environments may change as a population evolves (Jin *et al.*, 2013; Schaum & Collins, 2014). In the present study, after 1860 generations exposure to OA conditions, our population of *P. tricornutum* decreased its growth rate. The growth rate of the HCL population did not drop immediately, but was significantly reduced during generations 1733 ~ 1860. The decrease in growth rate of HCL populations might be partly related to decreased respiration, as dark respiration provides ATP and converts carbohydrates to carbon skeletons used for growth (Beardall & Raven, 2001). Short-term OA exposure

(within 20 generations) enhanced mitochondrial respiration of diatoms (present study; Wu *et al.*, 2010; Yang & Gao, 2012) due to a higher energy cost for cells to maintain intracellular acid-base homeostasis (Raven, 2011), and may lead to higher (Wu *et al.*, 2010) or unaltered (present study; Yang & Gao, 2012) growth rates compared to cells under ambient CO_2 conditions. After the long-term exposure to OA, physiological performance, such as changes in metabolic pathways (Jin *et al.*, 2015), might have reversed to save respiratory carbon loss and energy demand. The possibility of changes in selection on growth rate during long-term exposure to OA seems to be a pattern emerging in several studies across several taxa. At the very least, the overall effect of long-term growth under OA conditions may depend on the length of time spent in OA conditions, and may partially explain the variation in results of published studies.

P. tricornutum cells from populations cultured under elevated CO_2 for both long- and short-term showed higher relative photoinhibition than cells from the LCL population. This trend was consistent with previous results from the same strain of *P. tricornutum* (Wu *et al.*, 2010) and phytoplankton assemblages (Gao *et al.*, 2012b), indicating a higher sensitivity of cells grown under high CO_2 to high light stress. In addition to high light, phytoplankton cells and assemblages tend to be more sensitive to UVR under OA conditions (Sobrino *et al.*, 2008; Gao *et al.*, 2009; Sobrino *et al.*, 2009; Gao & Zheng, 2010), though complex interactions between OA and UV have been found in different species (Beardall *et al.*, 2014, and references therein). The down-regulation of photosynthetic machinery (Sobrino *et al.*, 2008) and decrease in

antioxidative enzyme activities (Pritchard *et al.*, 2000) under elevated CO_2 may be responsible for higher photoinhibition. The strategies that phytoplankton cells adopt under OA conditions involve sacrificing their tolerance to high light or UVR stress (indicated in present work by a higher photoinhibition ratio), while increasing investment to gain more from optimum light conditions (indicated in the present work by slightly higher rETR and effective photochemical quantum yield under growth light in the HC population).

Phytoplankton species are known to exhibit circadian variations in pigment production (Ragni & Ribera d'Alcalà, 2007), photosynthesis, and respiration (Bender *et al.*, 1987; Weger *et al.*, 1989; Chen & Gao, 2004; Halsey & Jones, 2015). While high CO_2 treated cells of the cyanobacterium *Trichodesmium* showed a different diurnal pattern of N_2 fixation from ambient CO_2 cells after short- (Eichner *et al.*, 2014) and long-term CO_2 exposure (Hutchins *et al.*, 2015), similar diurnal patterns of photosynthetic performance were observed in *Trichodesmium* (Kranz *et al.*, 2009) and a diatom, *Skeletonema costatum* (Chen & Gao, 2004) when grown under ambient and elevated CO_2 levels (although the absolute values were different). Nevertheless, there is still a possibility that diurnal physiological performance may differ under elevated CO_2 from ambient one over long-term exposures. While caution should be exercised when drawing general conclusions from the present work, here we described the phenotype of a population grown for > 1800 generations under high CO_2 with a phenotype that falls outside the documented range of short-term responses for this species.

The long-term consequences of OA on the main marine phytoplankton groups (cyanobacteria, diatoms, dinoflagelates, and coccolithophores) are beginning to be studied, and differences in the responses (direction and rate) of the main phytoplankton groups to OA on a long timescale will lead to shifts in marine community composition and thus the frequencies of some groups in ecosystems. Different responses to OA among phytoplankton groups might be caused by diverse abilities to assimilate dissolved inorganic carbon and adjust energy flow (Mackey *et al.*, 2015). Thus far, the evolutionary responses of coccolithophores and chlorophytes to elevated CO_2 often go in the opposite direction from the plastic (short-term) responses, in cases where an evolutinary response is detected (Collins *et al.*, 2014, and references therein). Cyanobacteria, on the other hand, have thus far shown evolutionary and plastic responses to OA that are in broadly similar directions of long- and short-term responses to OA, with some aspects of the high-CO_2-evolved phenotype being irreversible even after populations were returned to ambient CO_2 levels for 350 generations (Hutchins *et al.*, 2015). In term of diatoms, elevated CO_2 showed little effects on *T. pseudonana* after 100 generations of exposure, though this was perhaps too short a timescale to produce a measurable evolutionary response (Crawfurd *et al.*, 2011). Similarly, freshwater diatoms failed to evoke specific adaptations to elevated CO_2 after long-term exposure (Low-Décarie *et al.*, 2013). No phenotypic data other than growth rate are available to characterise patterns on the direction and magnitude of evolutionary responses in diatoms in this (Low-Décarie *et al.*, 2013) and another (Tatters *et al.*, 2013) studies. In the present study, our

population of *P. tricornutum* grown in OA conditions for 1860 generations showed phenotypic changes in a different direction from those seen in the population that was only exposed to OA conditions for about 20 generations. These phenotypic differences reflect different strategies adopted by the two populations of *P. tricornutum* cells. This indicates the potential for long- (probably evolutionary) and short-term (plastic) responses to OA that differ in direction for this species, and is in line with the observation that strategies that appear adaptive over the short term may not be those that evolve by natural selection over longer timescales (Schaum & Collins, 2014; Schaum *et al.*, 2016).

However, it is still worth noting that the present study did not measure evolutionary response to OA (which would have been revealed by switching HC selection line cells to the LC condition). Thus we cannot confirm whether the long-term response shown in the present study is a plastic or an adaptive response. Regardless of the attributes of the long-term response though, the present study provides evidence that the response of phytoplankton to OA may depend on the timescale for which they are exposed. The response of long-term OA exposure population in the present study may provide some clues and information for future evolutionary studies on diatom species. Only one population per treatment was used in the present study, which limits our ability to attribute the results to long-term OA exposure since replicates (populations) in one treatment may perform differently over the long-term period. Thus 4 or more independent replicates (populations) per treatment should be applied in future long-term experiments.

One interesting note in this study is that the value of many traits in the HCL population depends on when one stops the experiment. As with the fluctuations of growth and cell size over time, other traits may show either the same or different direction changes with those of HC population at different points in the 1860 generation timeline. Thus, the possibility of growth rate recovery in the HCL population cannot be reasonably excluded over even longer timescales. Even the physiological traits in the control population changed over time (as shown by growth rate and cell size). Indeed, if fluctuations in phenotype values are a general feature of long-term selection, then we expect that different experiments, each with a single arbitrary end point, might report different phenotypic values. Fluctuations in phenotype (such as competitive ability) have been documented in long-term evolution experiments (Barrick *et al.*, 2009), even where there is genetic evidence of continued adaptation. Thus, while long-term changes to traits need to be taken into account when predicting the responses of primary producers to OA, further studies that investigate and quantify how much these phenotypes are expected to fluctuate are necessary.

By culturing *P. tricornutum* in OA conditions for 1860 generations, we found that populations grown under long- and short-term elevated CO_2 differed from each other in growth and photosynthetic performance. The present study thus provides evidence that long- (probably evolutionary) and short-term (plastic) responses to OA may differ in direction for this species. The knowledge obtained from short-term experiments may therefore have limited scope for predicting how phytoplankton will

respond to the changing global ocean on a long timescale. Responses of phytoplankton to OA may depend on the timescale for which they are exposed. Differences in the responses (direction and rate) of the main phytoplankton groups to OA and other factors on a long timescale will influence ecosystem composition and primary production.

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Figure captions

Fig. 1 Growth rate (a) and cell size (b) ratios between *Phaeodactylum tricornutum* cells from HCL and LCL populations. The dashed lines indicate a ratio of HCL to LCL of 1. LCL, long-term ambient air exposure; HCL, long-term elevated CO_2 exposure. The values are means ± SD of triplicate cultures (n = 3). Growth rates and cell sizes prior to day 503 were reported in Li (2014) and shown here by randomly selected points from that data set.

Fig. 2 Growth rate (a), mean cell size (b), chlorophyll *a* (c), and carotenoid (d) contents of *Phaeodactylum tricornutum* cells from LCL (black bar), HC (white bar) and HCL (gray bar) populations. LCL, long-term ambient air exposure; HC, short-term elevated CO_2 exposure; HCL, long-term elevated CO_2 exposure. The measurements were determined after 1860 generations (1035 days) for LCL and HCL populations, and 20 generations for the HC population. The values are means ± SD of triplicate cultures from each population (n = 3). The different letters indicate significant differences among populations at $p < 0.05$.

Fig. 3 Dark respiration (a) and net photosynthesis (c) (μmol O_2 μg chl a^{-1} h^{-1}) per chl *a* for *Phaeodactylum tricornutum* cells from LCL (black bar), HC (white bar), and HCL (gray bar) populations. Dark respiration (b), and net photosynthesis (d) on a per cell basis (fmol $cell^{-1}$ h^{-1}) for the three populations. LCL, long-term ambient air exposure; HC, short-term elevated CO_2 exposure; HCL, long-term elevated CO_2

exposure. The measurements were determined after 1860 generations (1035 days) for LCL and HCL populations, and 20 generations for HC population. The values are means ± SD of triplicate cultures from each population (n = 3). The different letters indicate significant differences among populations at $p < 0.05$.

Supporting information captions

Table S1 Carbonate chemistry parameters of LCL, HC, and HCL media. These parameters were calculated from pCO_2 and pH with CO_2SYS software. The different superscript letters indicate significant differences among media at $p < 0.05$.

Table S2 Specific growth rates of LCL and HCL populations and the significance levels of differences in growth rate between the two populations for each measured growth cycle. Significant differences were analyzed using independent samples t tests, and the degrees of freedom were 4. Bold and underlined values show where there was significant difference in growth rate between the two populations.

Table S3 Mean cell sizes of LCL and HCL populations and the significance levels of differences in cell size between the two populations for each measured growth cycle. Significant differences were analyzed using independent samples t tests, and the degrees of freedom were 4. Bold and underlined values show where there was significant difference in cell size between two populations.

Fig. S1 Photosynthetic carbon fixation rates (pg $cell^{-1}$ h^{-1}) for *Phaeodactylum tricornutum* cells from LCL (black bar) and HCL (gray bar) populations at day 558 and 588. LCL, long-term ambient air exposure; HCL, long-term elevated CO_2 exposure. The values are means ± SD of triplicate cultures from each population (n = 3). The different letters indicate significant differences between populations at $p < 0.05$.

Table 1 Maximum (Φ_{PSII} max) and effective photochemical quantum yields (Φ_{PSII} eff), non-photochemical quenching (NPQ), apparent photon transfer efficiency (α), relative maximum electron transport rate ($rETR_{max}$), light saturation point (I_k), and relative photoinhibition ratio for LCL, HC, and HCL populations. LCL, long-term ambient air exposure; HC, short-term elevated CO_2 exposure; HCL, long-term elevated CO_2 exposure. The measurements were determined after 1860 generations (1035 days) for LCL and HCL populations, and 20 generations for HC population. The values are means ± SD of triplicate cultures from each population (n = 3). The different superscript letters indicate significant differences among populations at $p < 0.05$.

	Φ_{PSII} max	Φ_{PSII} eff	NPQ	α	$rETR_{max}$	I_k (μmol photons m^{-2} s^{-1})	Relative photoinhibition ratio (%)
LCL	0.670 ± 0.011^{a}	0.600 ± 0.004^{a}	0.044 ± 0.029^{a}	0.305 ± 0.013^{a}	122.5 ± 2.3^{a}	402.8 ± 23.5^{a}	11.4 ± 1.9^{a}
HC	0.672 ± 0.005^{a}	0.618 ± 0.002^{b}	0.048 ± 0.008^{a}	0.331 ± 0.015^{a}	122.1 ± 0.5^{a}	369.3 ± 17.2^{a}	16.9 ± 1.3^{b}
HCL	0.672 ± 0.002^{a}	0.594 ± 0.006^{a}	0.057 ± 0.006^{a}	0.302 ± 0.006^{a}	117.6 ± 1.6^{b}	390.9 ± 13.8^{a}	19.2 ± 2.0^{b}

1

Table 2 The population responses of representative parameters to elevated CO_2. HC, short-term elevated CO_2 exposure; HCL, long-term elevated CO_2 exposure. The values are calculated from the means of parameters from each population, and expressed as the fold difference compared to the LCL control.

	Growth rate	Cell size	Chlorophyll *a*	Net photosynthesis per chl *a*	Dark respiration per chl *a*	Net photosynthesis per cell	Dark respiration per cell
HC population	1.00	1.00	1.00	1.00	2.79	1.00	2.29
HCL population	0.87	0.96	1.33	0.64	1.00	1.00	0.58

Supporting Information

Methods

Photosynthetic carbon fixation

Sample (same density as culture) for determination of photosynthetic carbon fixation rate was inoculated with 5 μCi $NaH^{14}CO_3$ (ICN Radiochemicals). Samples were then incubated under growth conditions (150 μmol photons m^{-2} s^{-1}, 20 ºC) for 1 h. After the incubation, cells were gently filtered onto GF/F filters (25mm, Whatman) and the filters were placed into scintillation vials. Filters were exposed to hydrochloric acid fumes overnight, and dried at 50 ºC for 6 h. Scintillation cocktail was added to the vials before assimilated radiocarbon was counted by a liquid scintillation counter (Tri-Carb 2800TR, PerkinElmer).

Table S1 Carbonate chemistry parameters of LCL, HC, and HCL media. These parameters were calculated from pCO_2 and pH with CO_2SYS software. The different superscript letters indicate significant differences among media at $p < 0.05$.

Population	pCO_2	pH_T	DIC	HCO_3^-	CO_3^{2-}	CO_2	Total Alkalinity
	(μatm)		(μmol kg^{-1})	(μmol kg^{-1})	(μmol kg^{-1})	(μmol kg^{-1})	(μmol kg^{-1})
LCL	413±12[a]	8.02±0.02[a]	1943±74[a]	1750±63[a]	179±12[a]	13.4±0.4[a]	2206±88[a]
HC	997±15[b]	7.70±0.01[b]	2185±30[b]	2050±27[b]	102±4[b]	32.2±0.5[b]	2311±36[a]
HCL	1013±12[b]	7.70±0.01[b]	2187±58[b]	2054±53[b]	101±5[b]	32.7±0.3[b]	2310±64[a]

Table S2 Specific growth rates of LCL and HCL populations and the significance levels of differences in growth rate between the two populations for each measured growth cycle. Significant differences were analyzed using independent samples t tests, and the degrees of freedom were 4. Bold and underlined values show where there was significant difference in growth rate between the two populations.

Growth cycle	Specific growth rate		p value
	LCL	HCL	
1	1.32 ± 0.02	1.33 ± 0.01	0.571
2	1.33 ± 0.03	1.30 ± 0.02	0.281
3	1.27 ± 0.04	1.34 ± 0.01	0.060
4	1.30 ± 0.01	1.32 ± 0.01	0.069
5	1.39 ± 0.01	1.39 ± 0.03	0.949
6	1.34 ± 0.02	1.25 ± 0.01	**0.002**
7	1.26 ± 0.01	1.31 ± 0.01	**0.005**
8	1.31 ± 0.01	1.30 ± 0.01	0.288
9	1.34 ± 0.01	1.35 ± 0.02	0.656
10	1.32 ± 0.01	1.23 ± 0.01	< **0.001**
11	1.31 ± 0.01	1.27 ± 0.02	**0.018**
12	1.37 ± 0.01	1.32 ± 0.01	< **0.001**
13	1.28 ± 0.01	1.30 ± 0.02	0.103
14	1.31 ± 0.01	1.27 ± 0.01	**0.017**
15	1.34 ± 0.01	1.31 ± 0.01	**0.005**
16	1.32 ± 0.01	1.28 ± 0.01	< **0.001**

17	1.34 ± 0.01	1.29 ± 0.01	< **0.001**
18	1.33 ± 0.01	1.26 ± 0.01	< **0.001**
19	1.30 ± 0.01	1.30 ± 0.01	0.463
20	1.25 ± 0.01	1.23 ± 0.01	0.054
21	1.23 ± 0.02	1.28 ± 0.02	**0.036**
22	1.31 ± 0.03	1.35 ± 0.01	0.141
23	1.31 ± 0.03	1.30 ± 0.01	0.625
24	0.99 ± 0.01	1.12 ± 0.01	< **0.001**
25	1.39 ± 0.01	1.42 ± 0.01	0.089
26	1.21 ± 0.01	1.21 ± 0.02	0.935
27	1.28 ± 0.03	1.33 ± 0.02	0.078
28	1.31 ± 0.03	1.32 ± 0.03	0.568
29	1.30 ± 0.01	1.31 ± 0.02	0.384
30	1.31 ± 0.01	1.29 ± 0.03	0.325
31	1.29 ± 0.03	1.34 ± 0.02	0.050
32	1.25 ± 0.02	1.26 ± 0.03	0.503
33	1.22 ± 0.01	1.22 ± 0.01	0.826
34	1.23 ± 0.02	1.23 ± 0.02	0.928
35	1.28 ± 0.01	1.27 ± 0.02	0.824
36	1.27 ± 0.02	1.25 ± 0.02	0.280
37	1.27 ± 0.01	1.23 ± 0.01	< **0.001**
38	1.26 ± 0.01	1.28 ± 0.03	0.337
39	1.34 ± 0.01	1.32 ± 0.01	**0.036**
40	1.47 ± 0.01	1.50 ± 0.01	**0.013**
41	1.32 ± 0.02	1.32 ± 0.03	0.965

42	1.38 ± 0.02	1.24 ± 0.01	< **0.001**
43	1.25 ± 0.02	1.23 ± 0.01	0.287
44	1.33 ± 0.01	1.38 ± 0.01	**0.002**
45	1.36 ± 0.01	1.32 ± 0.01	**0.005**
46	1.08 ± 0.03	0.98 ± 0.03	**0.009**
47	1.32 ± 0.02	1.33 ± 0.01	0.497
48	1.24 ± 0.02	1.28 ± 0.03	0.162
49	1.31 ± 0.02	1.18 ± 0.03	**0.006**
50	1.26 ± 0.02	1.28 ± 0.02	0.187
51	1.33 ± 0.02	1.39 ± 0.03	**0.041**
52	1.32 ± 0.03	1.30 ± 0.02	0.390
53	1.21 ± 0.05	1.28 ± 0.04	0.142
54	1.25 ± 0.03	1.27 ± 0.02	0.333
55	1.27 ± 0.01	1.08 ± 0.01	< **0.001**
56	1.25 ± 0.01	1.14 ± 0.01	< **0.001**
57	1.29 ± 0.02	1.21 ± 0.01	**0.006**
58	1.12 ± 0.01	1.06 ± 0.05	**0.012**
59	1.40 ± 0.01	1.28 ± 0.01	< **0.001**
60	1.26 ± 0.02	1.21 ± 0.02	**0.016**
61	1.31 ± 0.03	1.22 ± 0.01	**0.012**
62	1.37 ± 0.01	1.19 ± 0.01	< **0.001**
63	1.35 ± 0.02	1.20 ± 0.01	< **0.001**
64	1.23 ± 0.05	0.96 ± 0.11	**0.017**

1

Table S3 Mean cell sizes of LCL and HCL populations and the significance levels of differences in cell size between the two populations for each measured growth cycle. Significant differences were analyzed using independent samples t tests, and the degrees of freedom were 4. Bold and underlined values show where there was significant difference in cell size between two populations.

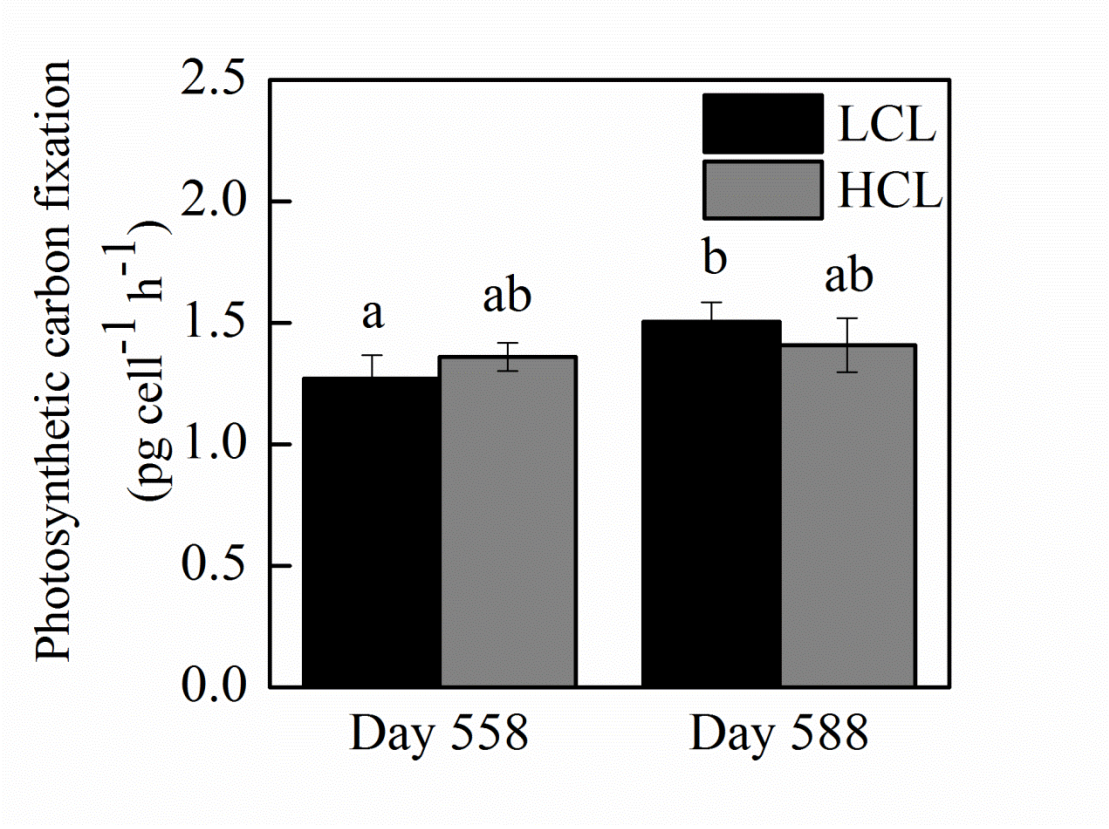
Growth cycle	Mean cell size		p value
	LCL	HCL	
1	5.04 ± 0.02	5.12 ± 0.08	0.205
2	4.77 ± 0.12	5.08 ± 0.01	**0.010**
3	5.08 ± 0.11	5.20 ± 0.22	0.129
4	4.96 ± 0.03	5.14 ± 0.11	0.054
5	5.02 ± 0.02	5.10 ± 0.07	0.124
6	4.62 ± 0.11	5.06 ± 0.02	**0.002**
7	4.96 ± 0.07	4.98 ± 0.02	0.657
8	4.91 ± 0.01	5.02 ± 0.04	**0.005**
9	5.25 ± 0.06	5.18 ± 0.03	0.195
10	5.08 ± 0.04	5.14 ± 0.05	0.175
11	5.03 ± 0.06	4.97 ± 0.01	0.206
12	5.23 ± 0.04	5.14 ± 0.02	**0.020**
13	5.00 ± 0.04	5.06 ± 0.02	0.056
14	5.17 ± 0.07	5.14 ± 0.02	0.531
15	5.12 ± 0.05	5.05 ± 0.02	0.107
16	5.10 ± 0.02	4.99 ± 0.01	**0.001**

17	5.15 ± 0.09	5.11 ± 0.03	0.487
18	5.23 ± 0.05	5.13 ± 0.01	**0.031**
19	5.45 ± 0.03	5.38 ± 0.01	**0.033**
20	5.29 ± 0.01	5.34 ± 0.01	**0.006**
21	5.38 ± 0.04	5.27 ± 0.01	**0.008**
22	5.46 ± 0.04	5.28 ± 0.01	**0.002**
23	5.39 ± 0.06	5.30 ± 0.02	0.059
24	5.59 ± 0.03	5.61 ± 0.02	0.284
25	5.50 ± 0.02	5.41 ± 0.02	**0.002**
26	5.38 ± 0.02	5.26 ± 0.01	< **0.001**
27	5.32 ± 0.08	5.24 ± 0.04	0.164
28	5.18 ± 0.18	5.11 ± 0.04	0.537
29	5.25 ± 0.03	5.11 ± 0.03	**0.007**
30	5.15 ± 0.01	5.03 ± 0.03	**0.002**
31	5.11 ± 0.01	5.03 ± 0.02	**0.004**
32	5.11 ± 0.06	5.01 ± 0.06	0.111
33	5.34 ± 0.17	5.04 ± 0.02	**0.039**
34	5.18 ± 0.05	5.05 ± 0.01	**0.009**
35	5.28 ± 0.02	5.20 ± 0.02	**0.011**
36	5.35 ± 0.03	5.21 ± 0.01	**0.002**
37	5.39 ± 0.05	5.18 ± 0.04	**0.006**
38	5.50 ± 0.03	5.28 ± 0.02	**0.001**
39	5.44 ± 0.05	5.33 ± 0.09	0.108
40	5.60 ± 0.04	5.37 ± 0.04	**0.002**
41	5.02 ± 0.06	4.84 ± 0.03	**0.012**

42	5.28 ± 0.02	4.90 ± 0.01	< **0.001**
43	5.12 ± 0.04	4.94 ± 0.02	**0.001**
44	5.22 ± 0.07	5.05 ± 0.09	**0.048**
45	5.07 ± 0.03	4.95 ± 0.02	**0.003**
46	4.82 ± 0.05	4.61 ± 0.02	**0.003**
47	5.02 ± 0.09	4.81 ± 0.04	**0.022**
48	5.16 ± 0.04	4.79 ± 0.02	< **0.001**
49	4.84 ± 0.03	4.66 ± 0.04	**0.002**
50	4.87 ± 0.01	4.70 ± 0.04	**0.002**
51	4.90 ± 0.03	4.77 ± 0.03	**0.009**

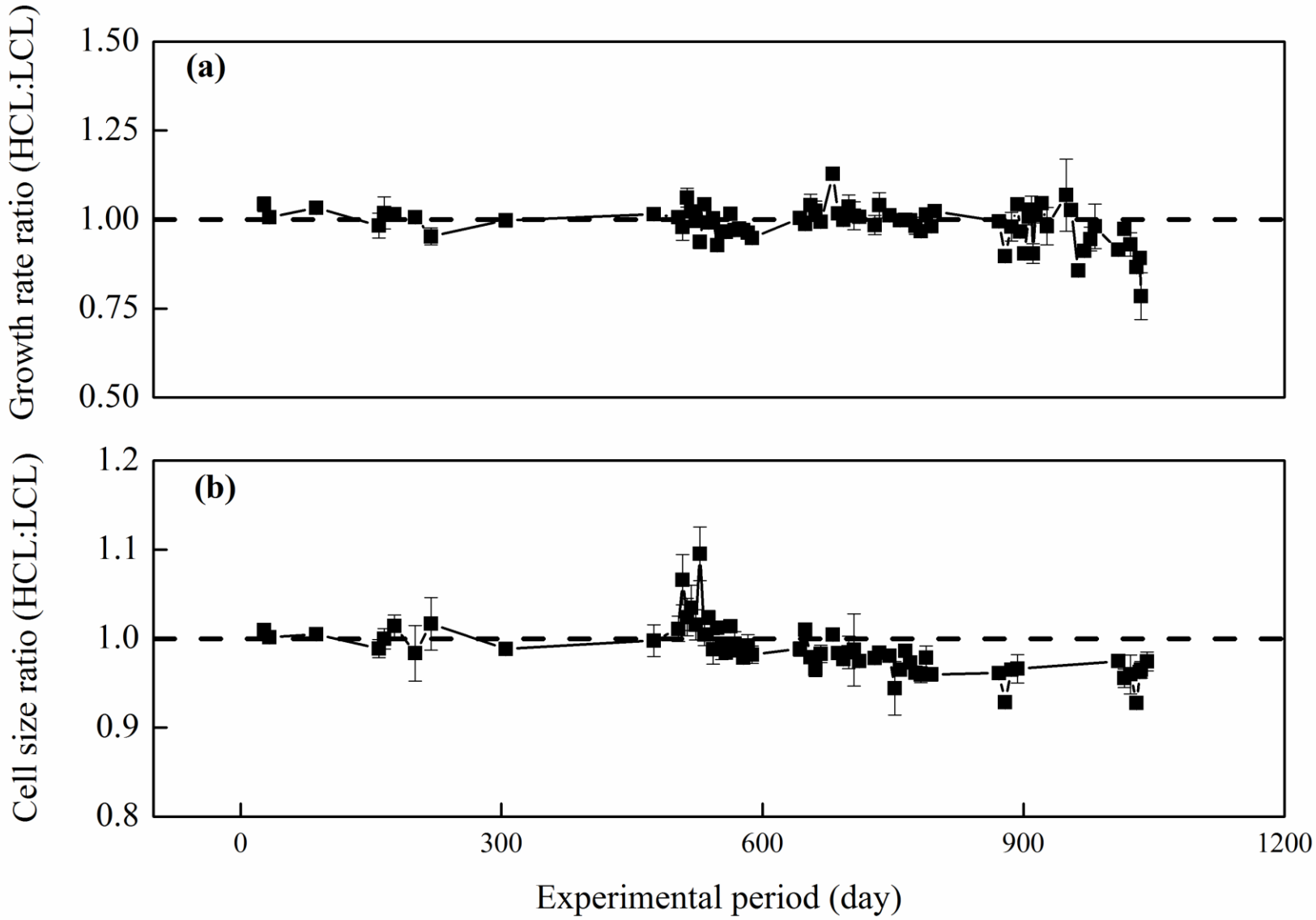
1

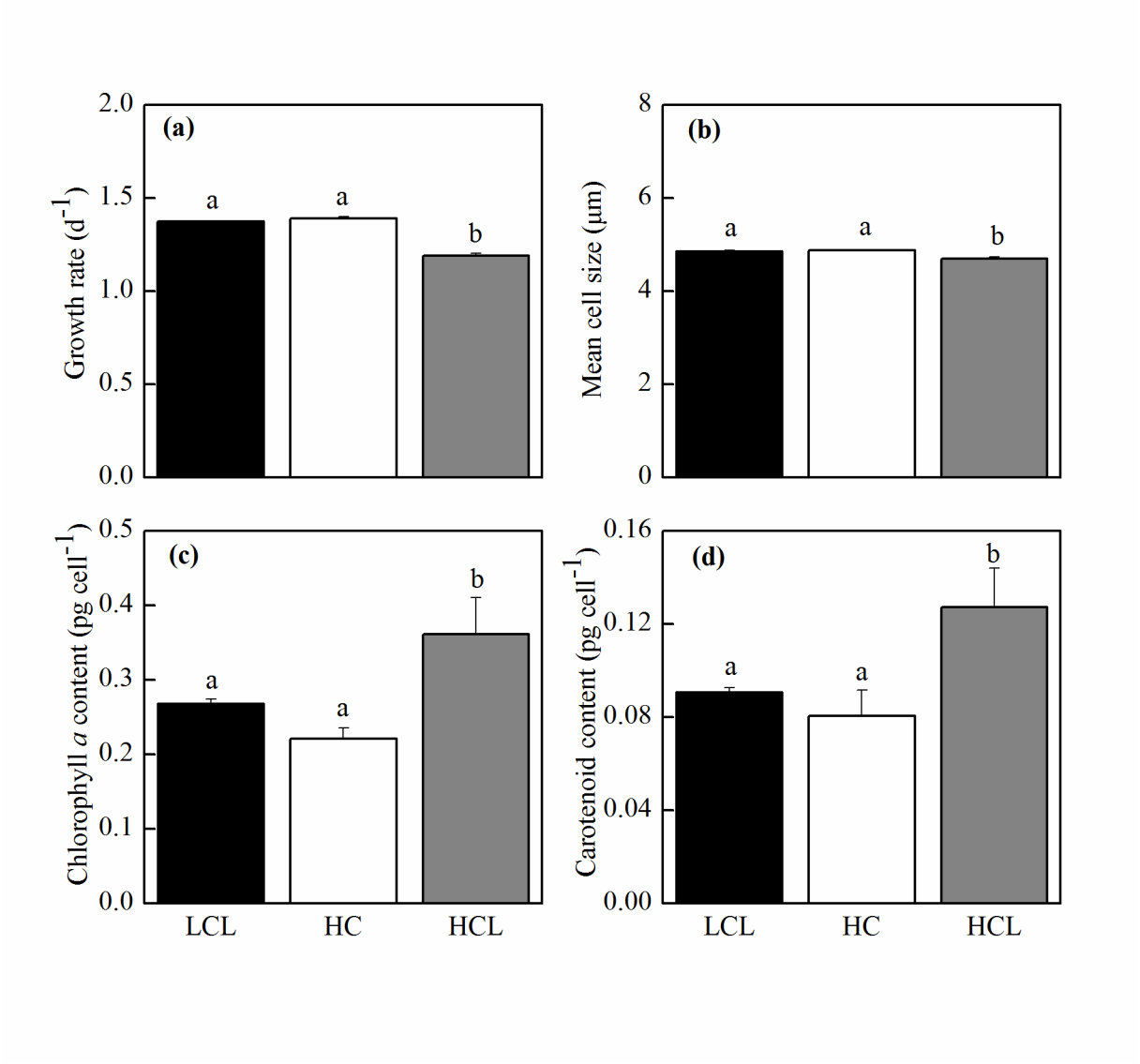
2

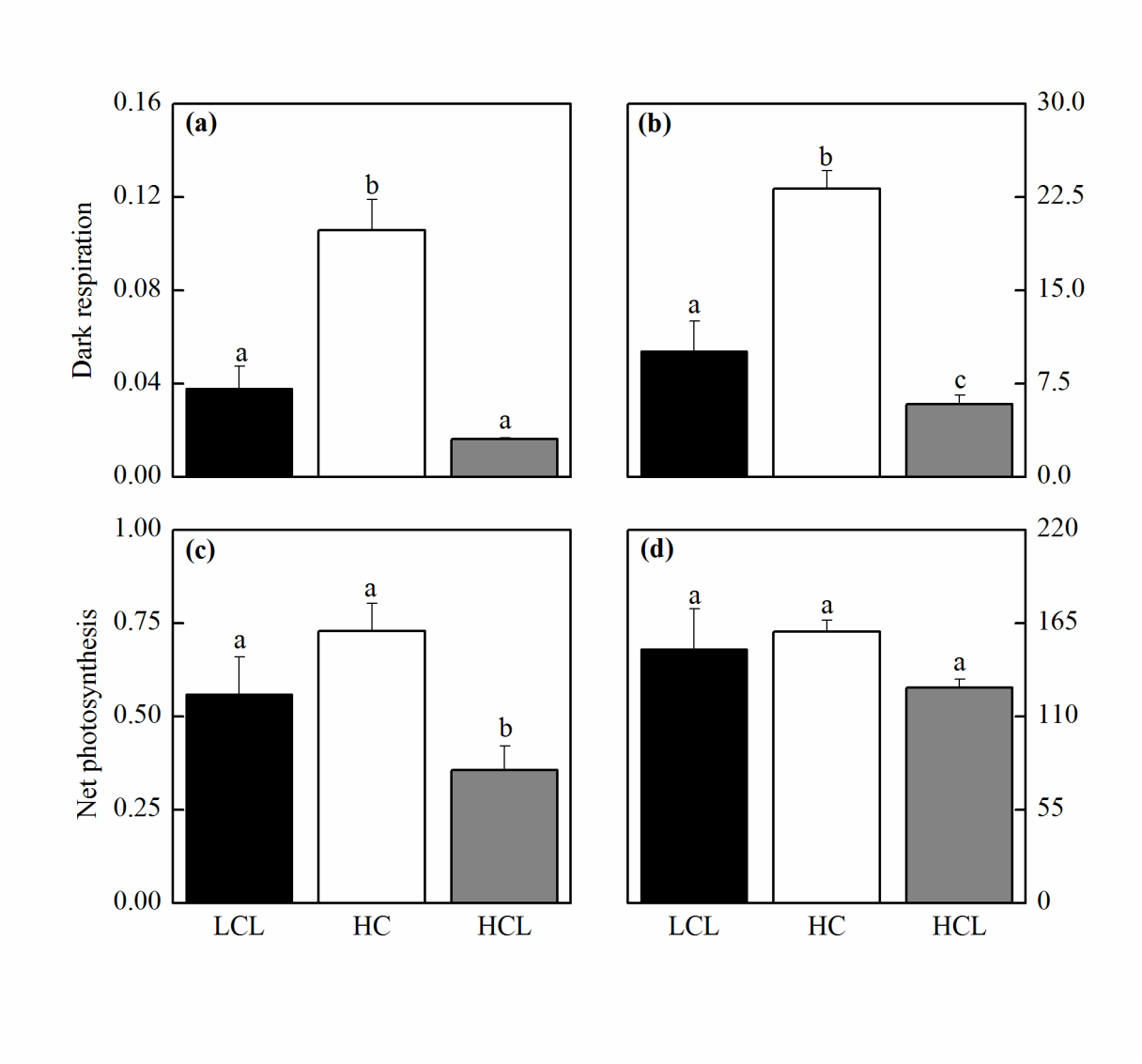


3

Fig. S1 Photosynthetic carbon fixation rates (pg $cell^{-1}$ h^{-1}) for *Phaeodactylum tricornutum* cells from LCL (black bar) and HCL (gray bar) populations at day 558 and 588. LCL, long-term ambient air exposure; HCL, long-term elevated CO_2 exposure. The values are means ± SD of triplicate cultures from each population (n = 3). The different letters indicate significant differences between populations at $p < 0.05$.


(a)
(b)
Growth rate ratio (HCL:LCL)
Cell size ratio (HCL:LCL)
1.50
1.25
1.00
0.75
0.50
1.2
1.1
1.0
0.9
0.8
0
300
600
900
1200
Experimental period (day)


(a)
Growth rate (d-1)
2.0
1.5
1.0
0.5
0.0
a
a
b
(b)
Mean cell size (μm)
8
6
4
2
0
a
a
b
(c)
Chlorophyll a content (pg cell-1)
0.5
0.4
0.3
0.2
0.1
0.0
a
a
b
LCL
HC
HCL
(d)
Carotenoid content (pg cell-1)
0.16
0.12
0.08
0.04
0.00
a
a
b
LCL
HC
HCL


(a)
(b)
(c)
(d)
Dark respiration
Net photosynthesis
0.16
0.12
0.08
0.04
0.00
30.0
22.5
15.0
7.5
0.0
1.00
0.75
0.50
0.25
0.00
220
165
110
55
0
a
b
a
a
b
c
a
a
b
a
a
a
LCL
HC
HCL
LCL
HC
HCL